PCT/GB03/02150

The Patent Office

INVESTOR IN PEOPLE

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED BUT NOT IN COMPLIANCE WITH RULE 17.1(a) OR (b)

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

REC'D 20 AUG 2003
WIPO PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears an amendment, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed R. Mahoney

Dated 14 July 2003

28JUN02 E729537-2 D00314
P01/7700 0.00-0214993.8

Patents Form 1/77

Patents Act 1977
(Rule 16)

PATENT OFFICE
A
28 JUN 2002
RECEIVED BY FAX

The Patent Office

1/77

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office
Cardiff Road
Newport
South Wales
NP9 1RH

1. Your reference — 45792 ~~P/63705.GB/AVISD~~

2. Patent application number *(The Patent Office will fill in this part)* — 0214993.8 — 28 JUN 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Marconi Applied Technologies Limited
One Bruton Street
London
W1X 8AG

Patents ADP number *(if you know it)* 78035130001

If the applicant is a corporate body, give the Country/state of its incorporation — **United Kingdom**

4. Title of the invention — **ELECTROCHEMICAL SENSING**

5. Name of your agent *(if you have one)* — **N. Hucker**

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Marconi Intellectual Property
Marrable House
The Vineyards
Great Baddow
Chelmsford
Essex CM2 7DS

REDDIE + GROSE
16 Theobalds Rd
London
WC1X 8PL

HL 25-2-03

Patents ADP number *(if you know it)* 28733000003

	Country	Priority application number *(if you know)*	Date of filing *(day / month / year)*
6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number			

	Number of earlier application	Date of filing *(day / month / year)*
7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application		

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
   a) any applicant named in part 3 is not an inventor, or
   b) there is an inventor who is not named as an applicant, or
   c) any named applicant is a corporate body.
   See note (d))

YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	(0)
Description	6
Claim(s)	(0)
Abstract	(0)
Drawings	(0)

10. If you are also filing any of the following, state how many against each item.

Priority documents	(0)
Translations of priority documents	(0)
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	(0)
Request for preliminary examination and search *(Patents Form 9/77)*	(0)
Request for substantive examination *(Patents Form 10/77)*	(0)
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Nerys Hucker Date 28/06/02

12. Name and daytime telephone number of person to contact in the United Kingdom: **Nerys Hucker 01245 493 493 ext 3122**

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Electrochemical Sensing

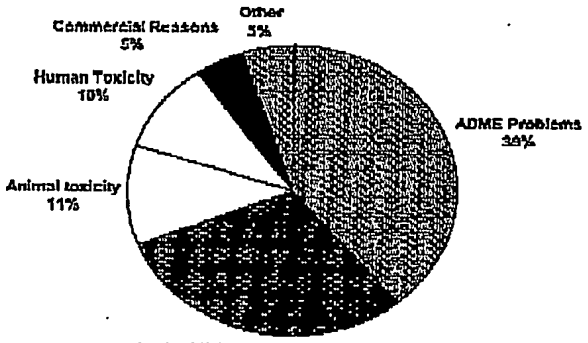
Summary

A key area of interest in the pharmaceutical industry is the prediction of how drugs are metabolised in the body. One of the main drug metabolism processes, phase I oxidation, is mediated by either the cytochrome P450 [CYP] or flavin monooxygenase (FMO) families of enzymes. The reactions catalysed by these enzymes can be driven electrochemically, and the reaction progress may be monitored using simple electrode systems. This makes the CYPs, and the functionally-related FMOs ideal candidates for electrochemical sensing.

Background

The single most important decision in the drug design process is made when selecting which of the lead compounds identified during the research programme are to be passed into the development pipeline. About 90% of development candidates fail to become marketed drugs, for a variety of reasons (see figure, below). The cost of a development programme is extremely high (typically £50M per molecule), and so the selection of development candidates has a high financial penalty if made incorrectly. For this reason, there is intense interest within the pharmaceutical industry for effective means to 'fail drugs early', identifying compounds that are unlikely to make it to market before vast expenditure is incurred.

Causes of Attrition in Drug Development



Data shown for 198 potential drug candidates which failed during the development stage, taken from Kennedy, T. (1997) Drug Discovery Today 2, pp.436-444

The greatest single reason for a drug candidate to fail during development is for it to show unacceptable characteristics when introduced into live animals or humans. The collective term for these characteristics is ADME/Tox (absorption, distribution, metabolism, excretion and toxicity), covering how well a molecule enters the body, is distributed among the various tissues, is biochemically processed, and then eliminated in the bile or urine, as well as any unexpected toxicological effects that may be uncovered during the development and clinical trials programmes. ADME/Tox prediction is a current area of intense research, and will be an expanding market over the next 3-5 years.

Electrochemical sensing has a role to play in several areas within the ADME/Tox area, since many of the drug metabolism processes involve changes in redox potential. It therefore provides a means to quantify drug molecules, and their metabolic effects, in the context of a whole tissue or body fluid sample. In particular, it provides a sensitive and cost-effective means to follow the metabolic processing of drugs either at the single-enzyme, or whole organ level.

The drug-metabolising enzymes [DMEs] are a diverse group of proteins that are responsible for detoxifying a vast array of xenobiotic compounds ('foreign molecules') including drugs, pesticides and environmental pollutants. Most have an extremely broad substrate specificity: some individual members of the cytochrome P450 [CYP] and flavin monooxygenase [FMO] families are known to metabolise more than 50 structurally diverse compounds. Understanding the structure-activity relationships for the DMEs and their substrates is an important area of research that impacts on pharmacology, toxicology, and basic enzymology. In particular, the ability to predict whether a molecule is likely to be processed by CYPs in the body is of crucial importance in selecting candidate drug molecules for pharmaceutical development.

Conceptually, the DMEs are divided into two groups. Oxidative drug-metabolising enzymes, which include CYPs and FMOs, catalyse the introduction of an oxygen atom into substrate molecules, generally resulting in hydroxylation or demethylation. These enzymes are redox-driven, and the reactions they catalyse may readily be followed electrochemically. The conjugative enzyme families, which include the UDP-glycosyltransferases, glutathione transferases, sulphotransferases, and N-acetyltransferases, catalyse the coupling of endogenous small molecules to xenobiotics. This usually results in the formation of soluble compounds that are more readily excreted. The conjugative enzymes are not redox-driven, and are therefore not particularly suitable for electrochemical sensing. Other, as yet unidentified DMEs may also be found as a result of the human genome project. For example, the recently-discovered CYP3A34 has now been shown to be expressed in certain body tissues, although its precise function is currently unclear. The discussion here is therefore not limited to the enzymes explicitly mentioned in the text.

The CYP and FMO oxidative drug-metabolising enzymes are of particular interest as the biosensor components of an electrochemical device since the electrons they require to drive the reactions can be supplied by direct charge transfer from electrodes in a bioreactor chamber. Indeed, in such a device there is no requirement for additional biological or chemical components such as the cofactors and ancillary oxidoreductase enzymes that are necessary for driving the reactions in a conventional *in vitro* assay.

Proposed Biosensor Components

Cytochrome P450

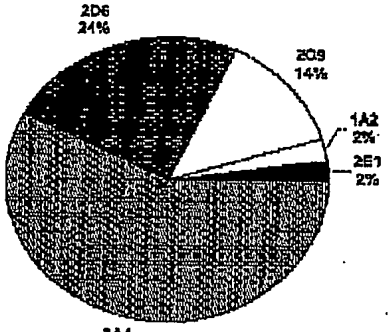
3D molecular structure of CYP2C9 showing the haem group, active-site iron atom and a bound substrate.

CYP enzymes catalyse the initial step in the biotransformation of xenobiotic compounds, including most drugs (a process referred to as first-pass or Phase I metabolism). These enzymes are members of a large family of mixed-function oxidases which typically introduce an oxygen atom into substrate molecules, hence facilitating further metabolic processing to break the compound down. More than fifty CYP isozymes are known to exist in humans and they have been classified into 17

families and 39 subfamilies. In the standard nomenclature, the family is designated by a number, a letter designation for the subfamily, and a second number that identifies the individual member of that subfamily.

In humans and animals, the bulk of drug metabolism is carried out by just a few members of the CYP1, 2, and 3 families and occurs primarily in the liver, which contains the highest concentration of CYP in the body. The figure below shows the percentage of drugs metabolised by the different CYP families. CYP3A4, 2D6, 1A2 and 2C9 are responsible for most of the drug metabolism by CYPs in humans, so are considered the most interesting from a drug screening point of view.

Drug Processing by the Major CYP Isozymes



Data shown for 308 drugs which are metabolised by known CYP enzymes, taken from Parkinson, A (1996) Toxicology, McGraw-Hill

The oxidation of organic molecules by CYPs is quite complex, but the overall reaction can be simplified to the following equation:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

An electron from the cofactor NADPH (a common electron-transfer molecule) is transferred to a haem group within the CYP, where the activation of molecular oxygen occurs. Substrates (represented as R in the above equation) react with one of the oxygen atoms whilst the other is reduced to water, requiring a second electron. Several studies have shown that the electrons necessary to drive this reaction can be supplied electrochemically, with direct charge transfer coming from electrodes in an anaerobic bioreactor. In this case, there is no requirement for the NADPH cofactor. CYP enzymes are therefore ideal candidates for incorporation into an electrochemical sensor for predicting drug metabolism.

Flavin Monooxygenases

Flavin monooxygenases, like the CYP enzymes, catalyse the oxidation of organic compounds using molecular oxygen and NADPH as the source of electrons for the reduction of one of the oxygen atoms. However, they are mechanistically distinct from the CYPs in that they react with oxygen and NADPH in the absence of substrate to adopt an activated state within the cell, and an interaction with a nucleophilic group such as an amine, thiol, or phosphate is all that is required for completion of the catalytic cycle.

The capacity to remain stable whilst poised in an activated state is a possible explanation for the extremely broad substrate specificity of the FMO isozymes. It has been proposed that essentially all of the energy required for catalysis is captured in the oxygen-activated intermediate, and that alignment or distortion of

the substrate molecules is not required, unlike most other enzymes. It follows that the active sites of FMOs are much less sterically defined than for other enzymes, allowing a wide variety of molecules to act as substrates. FMO3 is the most abundant form in human liver and is believed to be the dominant member of this enzyme family in terms of overall drug metabolism.

As for CYPs, it is possible to drive the FMO-mediated reactions by supplying electrons electrochemically, and therefore these would also be ideal candidates for incorporation into an electrochemical sensor device for predicting drug metabolism.

Protein-Electrode Interactions

Immobilised Proteins

One of the most important aspects of driving an enzyme-catalysed reaction electrochemically is the efficient transfer of electrons from the electrode(s) to the catalytic site within the enzyme. One way to maximise this transfer is to immobilise the enzyme at the surface of the electrode. Although a system involving a solubilised enzyme could be designed, early success is most likely by using surface-immobilised enzymes.

Covalently-modified Electrodes

The surface of an electrode of, for example, metal (typically, though not exclusively gold) or graphite, can be modified by the covalent addition of chemical groups to make it more amenable for the transfer of electrons to proteins. One technique involves the use of organothiolate compounds (containing an SH group) in conjunction with a gold electrode. The thiol group forms a strong bond to the metal surface, with the rest of the molecule providing suitable functional groups for interacting with the protein.

Microporous Electrolyte Membranes

These are mechanically and chemically stable polymer gels with high ionic conductivity, coating the surface of an electrode in the form of a thin layer. The polymers comprising the gel should be chosen to provide a suitable environment for trapping the proteins within their matrix, such as a high proportion of carboxylic acid groups (for proteins with many positively-charged surface residues), amine groups (for proteins with many negative charges at the surface), or aliphatic groups (for proteins with largely hydrophobic surfaces).

Lipid Membranes

Natural CYP enzymes are usually found attached to biological membranes, since they almost exclusively contain a region which acts as an anchor within a phospholipid bilayer. Indeed, the CYPs used in analytical laboratories are generally modified to remove this anchor domain, thus allowing the enzyme to be solubilised. The affinity of CYPs for lipid bilayer membranes provide a means of anchoring them at the surface of an electrode. Suitable membranes may be constructed using long-chain fatty acids, lipids, or similar molecules, deposited on the surface.

Application Areas

Ideally, a drug development team would like to have a detailed picture of the pathway and kinetics of a compound's metabolism in humans, including possible side effects such as CYP induction/inhibition and the generation of toxic metabolites, before beginning clinical trials. Gathering as much of this data as possible usually involves a combination of increasingly targeted assay systems. Whole animals are often used for initial toxicological assessment and the outcome of these experiments can prevent a compound from entering the next phase even before any metabolism work is done. Such studies are currently examined using cultured liver cells, live animals or liver slices in combination with a variety or analytical methods to determine the overall metabolic profile. Most recently, such assays are performed using microsomes, synthetic cells comprising isolated enzymes held in an artificial membrane.

Even with the application of increasingly sophisticated analytical methods, there are obvious difficulties in using animals, cells, or cell fractions to obtain information on the specific biochemical events that comprise a compound's metabolism. Advances in the molecular genetics and biochemistry of the DMEs, and the need for greater efficiency in the drug discovery process are driving the development of new *in vitro* methods based on isolated DMEs. These methods have been used for screening thousands of compounds, and are amenable to integration into the early phases of the drug discovery process. Some of the ways in which recombinant CYPs have been used for *in vitro* metabolism studies and the rationale for these are described in the following sections. The same general approaches can be applied to other DMEs such as the FMOs, but in most cases the methods are not nearly as well developed as they are for the CYPs.

Isozyme Identification

An identification of the major enzyme(s) involved in your specific drug's metabolism is perhaps the most important component of early studies. Once this is known, pharmacokinetic [PK] studies (see below) are done to obtain K_m (an approximate measure of the affinity of the enzyme for the substrate) and V_{max} (how fast the enzyme can process substrate molecules). Together, these parameters are used to estimate *in vivo* clearance rates, a key determinant of therapeutic efficacy. Knowledge of the metabolism rate by a specific enzyme may alert the drug discovery team to potential pharmacogenetic problems or drug-drug interactions.

Genetic differences in CYP levels are a major cause of individual variability in response to therapeutics. For example, roughly 8% of the Caucasian population are poor metabolisers of 2D6 substrates and can experience serious side effects when administered normal doses of drugs that are metabolised primarily by this isozyme. Furthermore, some drug-drug interactions can cause serious side effects or even fatal conditions such as drug-induced arrhythmia. The identification of which enzyme is primarily responsible for the metabolism of a drug aids in the design of effective clinical studies used for assessing possible drug interactions. A panel of CYP and FMO enzymes used as biosensors would enable the degree of processing of a new drug by each isozyme to be accurately quantified.

Determination of Kinetic Parameters

Undesirable PK characteristics are frequently a factor in the failure of compounds in preclinical studies. The goal of *in vitro studies* is to determine the key PK parameters (K_m and V_{max}) for a compound with each CYP isozyme in order to obtain an estimate of the overall *in vivo* clearance rate. The problems with attempting to obtain accurate kinetic data from crude enzyme preparations such as microsomes include metabolism of the substrate by more than one isozyme, further modification of products (e.g. conjugation), consumption of NADPH by contaminating redox enzymes, and binding of substrates or products to cell

proteins or other macromolecules. From an enzymologist's point-of-view, the only way to obtain accurate kinetic data is with isolated enzyme systems. Isolated CYP enzymes used as biosensors would provide this capability.

High-Throughput Screening

A large number of pharmacologically active compounds synthesized in the discovery phase of pharmaceutical R&D are rejected because they interact with the metabolism of existing therapeutic drugs or because they have poor bioavailability caused by rapid metabolism. In many cases, this is because the compounds are either substrates or inhibitors of one or more CYP isozymes. CYPs and other DMEs are generally assayed by isolation and quantification of the metabolites produced from the parent compound. In most cases, this involves chromatographic techniques (usually HPLC) and in some cases phase separations. There are two major drawbacks to these assay methods. First, the need to isolate the reaction products makes the methods too cumbersome and time consuming for use in any type of high-volume assay and precludes the collection of continuous kinetic data. Second, measurement of metabolites requires use of different assay methods for every substrate, raising an obvious technical barrier to screening diverse compounds for metabolism. A universal assay method would be ideal in that it would allow direct quantification of metabolism rates for any substrate, allowing the determination of the key pharmacokinetic parameter (calculated as V_{max}/K_m) for diverse compounds in a high-throughput screening [HTS] format. The intuitive approach for achieving this is to monitor NADPH consumption, which theoretically should be stoichiometric with substrate turnover. However, this has not proven practical because the coupling between NADPH consumption and substrate turnover is variable between different substrates and is frequently as low as 20-30%. Measurement of oxygen consumption suffers from the same drawback; a significant percentage of the total oxygen consumed is diverted into reactive oxygen intermediates rather than metabolite and water.

For these reasons, the main approach that is currently used for screening is competitive inhibition assays, in which inhibition of a probe substrate turnover by the test compound is used to identify potential substrates and inhibitors. The hits from these competitive inhibition screens must be further evaluated to determine whether they are inhibitors or substrates for the indicated isozyme. A number of approaches have been developed for high-throughput screening of CYP inhibition. These techniques include rapid phase separation methods for isolating radiolabeled CYP 2D6 metabolites, development of robotically controlled, multi-column HPLC separation systems to assay testosterone metabolism by CYP 3A4, the use of novel chromogenic reagents for quantitation of formaldehyde formation during CYP-dependent demethylation reactions, and rapid LC/MS approaches for metabolite analysis. However, all of these approaches include relatively cumbersome post-reaction separation steps that limit their usefulness in an HTS format. A lab-on-a-chip style biodetector able to follow CYP mediated reactions at the pharmacokinetic level would not require these separation steps, and so would offer substantial benefits over the current HTS technologies.